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(54) Title: REPELLENT COMPOSITIONS CONTAINING FLAVONOID ALDEHYDES

(57) Abstract

Repellent compositions which contain falvonoid aldehydes such as cinnamic aldehyde, α-hexyl cinnamic aldehyde and/or coniferyl aldehyde are provided, together with methods for their use as repellents for pests including flies, cockroaches, aphids, silverleaf white flies, mosquitos, ticks, fleas, leafhoppers, thrips, two-spotted spider mites, snails, slugs, biting midges, earwigs, and moths.

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REPELLENT COMPOSITIONS CONTAINING FLAVONOID ALDEHYDES

INTRODUCTION

Technical Field

This invention relates to flavonoid aldehydes as pest repellents. The invention is exemplified by the use of cinnamic aldehyde or alpha hexyl cinnamic aldehyde as a mosquito repellent and for repelling agricultural pests such as aphids and thrips.

Background

In many countries today, diseases such as malaria, vector-borne hemorrhagic fevers, cockroaches allergies, filth fleas, bubonic plague, ticks viruses, rickettsiae, spirochate bacteria, snails-schistosomiasis, and sand fly fever are still responsible for serious illnesses and numerous deaths among inhabitants. The ever-growing concern for the protection of endangered species and the downward trend in availability of the broad spectrum pesticides for public health are forcing scientists to look for other economical means of providing protection from vectors of disease. Moreover, the cost of pesticides often is too high for many of the less developed nations and the increasing resistance to these compounds by vector populations is a growing problem.

The use of repellents is an excellent alternate means of providing relief when other conventional vector control methods are not feasible. For mammalian vector targets, repellents properly applied to the skin and/or clothing are an inexpensive and practical means of reducing the biting activity of hematophagous arthropods and for the prevention of vector disease transmission. Repellents are effective against a wide range of disease vectors, whereas a separate vaccine must be developed for each disease. Moreover, only a few vaccines are effective against vector-borne diseases. Diseases spread by vectors also affect plants. For example, Dutch Elm Disease has destroyed millions of elm trees across the United States. The disease is caused by a fungus which is spread from tree to tree by a particularly species of insects attracted to the elms. Current methods of prophylaxis have had

only limited success. A need exists for an effective prophylaxis for this destructive plant disease.

Retractions of the use of repellents in vector control and disease prevention usually center on questions of safety and cost. For example, one of the more extensively used repellents was the 6-2-2 repellent which contained dimethyl phthalate, ethyl hexanediol, and Indalone in the proportion 6:2:2. Dimethyl phthalate and Indalone are still in limited use, but in 1991, the U.S. Environmental Protection Agency canceled all registrations of ethyl hexanediol at the request of the manufacturers concerned. This action was taken because of new information on possible adverse effects on fetal development.

As another example, one of the most effective mosquito repellents is DEET (N.N-diethyl-1.3-methylbenzamide). This material virtually eclipsed other repellents for topical use, and it remains the principal repellent in use today, nearly 40 years after its discovery. However, in recent years permethrin, a synthetic derivative of pyrethrum, has largely replaced DEET for use on clothing and other fabric items. As a repellent, DEET is highly effective, but it may also cause allergic and toxic effects, especially when used on the skin repeatedly in high concentrations. Repellent formulations containing 90-99% DEET are considered high concentrations, whereas repellent formulations containing 50% or less DEET are considered as effective as a concentration of 100%. A concentration of 33% DEET is effective in providing 10-14 hour protection. However, for products containing even low concentrations of DEET, it is recommended that the skin be cleaned with mild soap and rinsed with water as soon as the repellent is no longer needed, in order to minimize possible adverse reactions. These recommendations frequently are impractical in third world countries and for military use.

Repellent compositions exist for topical application to a mammal, as well as to repel insects from entering a dwelling or other area. However, the safety of many of the topical compositions has been questioned. Moreover, many of the topical compositions are of limited effectiveness, especially in areas of severe infestation with insects. Treatment for external insect infestations of a mammal, such as lice or crabs, often involves topical application of harsh toxic insecticidal compositions to skin or scalp. Irritation often develops, and adverse health effects from long-term use are also known. A need exists for a safe, effective topical repellent composition for a mammal. Repellent compositions for the prevention of entry of insects are similarly ineffective. Many of the known such repellent compositions also are not safe for use in enclosed spaces due to their high toxicity, especially

where children and pets may come into contact with them. A need exists for a non-irritating, non-toxic, effective repellent composition.

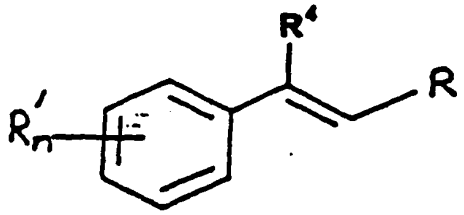
Insect infestations of trees and other woody plants destroy millions of ornamental and agricultural trees every year. Current treatments are only partially successful, and may render a crop of agricultural products worthless due to their persistent toxicity. Thus, a need also exists for a relatively non-persistent, effective means for repelling insect infestations of trees and woody shrubs.

Relevant Literature

USPN 5,093,326 discloses repellent compositions which include an ozonized unsaturated hydrocarbon, including terpenes. USPN 5,365,017 discloses preparation of a transgenic plant having increased levels of cycloarterol. Publications relating to repellant formulations include Reifenrath *et al.* (1989) *J. Am. Mosquito Control Association* 5: 45-61 and Reifenrath (1995) *Cosmetics & Toiletries Magazine* 110: 85-93.

SUMMARY OF THE INVENTION

The present invention provides repellent compositions which contain flavonoid aldehydes and methods of using these compositions. The repellent compositions contain a compound which has a formula shown in (1) below:



(1)

wherein R represents $-CH_2OH$ or -CHO; n is an integer from 0 to 3; and each R_1 independently represents OH or an organic substituent containing from 1 to 10 carbon atoms and from 0 to 5 heteroatoms, wherein the total number of carbon and heteroatoms in all R_1 substituents of said compound is no more than 15; and R^4 represents hydrogen or an organic constituent containing from 1 to 10 carbon atoms. These compounds include natural compounds, such as cinnamic aldehyde. Also of interest are alpha substituted aldehydes, such as alpha hexyl cinnamic aldehyde (HCA). In use, the compounds are applied to a surface, such as skin, clothing, bark, habitat components and the like, from which it is

desirable to repel insects and other pests. The invention finds use, for example, in the prevention of disease and infection which can result from contact with a disease-carrying insect vector or other pest vector.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Methods and compositions are provided for obtaining and/or maintaining an area substantially free of insects and other pests. Mammals, birds, fish and their habitats, as well as seeds, seedlings, plants, and plant parts such as fruit substantially free of pathogenic organisms such as fungi, insects and other pests, as well as viruses, bacteria, spirochetes, and other disease-causing organisms, and sap-sucking insects are provided together with a method to repel pests and disease-causing organisms. A surface of interest is contacted with flavonoid aldehyde in an amount sufficient to repel an insect or other pest. The amount of repellent that is applied will depend in part upon the nature of the surface, and to some extent upon the formulation and the specific compounding used and, therefore, must be empirically determined for best results with a particular insect or other pest.

The compositions and methods of the subject invention offer several advantages over existing compositions and methods. A major advantage is that the formula components are generally regarded as safe (GRAS) and approved for food use. For example, a number of the aromatic aldehydes which may find use in the subject invention, such as α-hexyl cinnamic aldehyde (HCA), cinnamaldehyde, and vanillin are GRAS synthetic flavoring agents (21 CFR §172.515). HCA was in public use before the 1950's and today is widely used in consumer preparations (soaps, detergents, creams, lotions, perfumes) (Monographs on fragrances raw materials. *Food Cosmet. Toxicol.* 12: suppl., 915, 1974). HCA was granted GRAS status by FEMA (Flavoring Extract Manufacturers' Association. Survey of flavoring ingredient usage levels. No. 2569. Fd. Technol., Champaign, 19: (part 2) 155, 1965) in 1965 and is approved by the US FDA for use in food (21CFR121.1164). The Council of Europe (1970) (Council of Europe. Natural and Artificial Flavouring Substances. Partial Agreement in the Social and Public Health Field. Strasbourg, List A(1), Series 1, no. 129, p. 55, 1970) included HCA in the list of admissible artificial flavoring substances at a level of 1 ppm.

Surfactants which can be used as emulsifiers in the subject formulations, such as the Tweens (polysorbates) also already are used as food additives, as is saponin (which also has GRAS status). In addition, formulation residuality can be managed. This is of great benefit for integrated pest management programs with beneficial insects because short term residuals

can be obtained. The long term control of pathogenic organisms results in a healthier plant and an improved yield of produce by the host plant as compared to nontransgenic plants. The aromatic aldehydes in particular have positive organoleptic and olefactory properties which in some cases can improve the flavor and/or smell of treated products and eliminate the unpleasant odor associated with many pest repellants. The odor of α-hexyl cinnamic aldehyde (HCA), for example, is described as floral or jasmine-like with some herbaceous character (Technical Data Sheet).

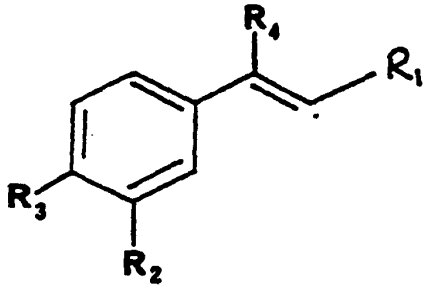
For plants, the active ingredients can be made by the plant following introduction of the gene(s) responsible for synthesis of the various aldehydes into the plant cell genome. The use of transgenic plants rather than topical application of repellent to the plant decreases the likelihood of any adverse side effects to field workers, or to animals, fish or fowl which ingest the tissues or parts of the plants, since many of the compounds of formula (1), in particular those of formulas (3) and (4), are GRAS food additives. In addition, the subject invention overcomes the failure of current pesticides to translocate, for example, to roots for treatment of phylloxera. Also, prevention of infestation by repelling the vector which carries diseases, or damages the target for the pest, significantly decreases the number of target animals or plants which will succumb to disease carried by the pest or be damaged by the activities of the pest, such as, for example, the damage done by female medflies as they oviposit on fruit.

When applied to animals, including humans, the subject formulations are non-toxic and non-irritating to the skin at the concentrations used. For example, α-hexyl cinnemaldehyde (HCA) has an oral LD_{50} of 3.1 g/kg in rats and a dermal LD_{50} of greater than 3 g/kg (Moreno, O.M. Report to RIFM, March 24, 1971). HCA was found to be moderately irritating when the neat compound was applied to intact or abraded rabbit skin for 24 hours under occlusion (Moreno). When tested at 12% in petrolatum, HCA produced no irritation after a 48 hour closed-patch test on human subjects and produced no sensitization in a maximization test carried out on 25 human subjects (Kligman (1966) *J. Invest. Dermatol.* 47: 393). HCA at 20% in diethylphthalate produced no positive reactions in a repeated insult patch test conducted on 100 human subjects. Jimbo tabulated allergenicity data found in the literature for 18 fragrance compounds. While cinnamic aldehyde had a positive reaction from the human maximization test, HCA was negative in the test. Patch test results of 2% HCA on 100 eczema and dermatitis patients were negative (0 out of 100). Of 4 patients sensitive to 2% cinnamaldehyde, none were found to cross react with 2% HCA. The skin

sensitization frequently reported for cinnamaldehyde is probably initiated by reaction of amino groups on proteins with the aldehyde functional group. Substitution of bulky alkyl groups in the alpha position (*e.g.* the hexyl group of HCA) relative to the aldehyde group can reduce this reactivity by creating steric hindrance as well as reducing the electrophilicity of the aldehydic carbon. Alpha-substituted cinnamaldehydes, to which skin sensitization is uncommon, react very slowly or not at all with amines in comparison with cinnamaldehyde. In studies using the maximization test in guinea pigs, Senma and coworkers report a tendency that as the number of hydrocarbons of alkyl groups replacing the alpha-hydrogen in cinnamaldehyde increased, the rate of sensitization reaction declined.

The subject formulations also provide for effective control of multiple organisms, such as both fungi and insects. The compounds also have been reported to have inhibitory activity against *C. botulinum* spore germination (Bowles and Miller, *G. Food Protection* (1993) 56: 788-794). This multi-target efficacy reduces the need for application of multiple agents to a plant or animal to be protected, and substantially eliminates the need for application of pesticides. In particular situations, such as where an insect damages an animal or a plant part or tissue and a secondary fungal or bacterial disease develops, this aspect of the invention is particularly advantageous.

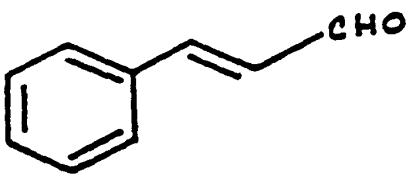
The general formulation is as shown in formula (1) above. A preferred formulation is shown in formula (2) below:



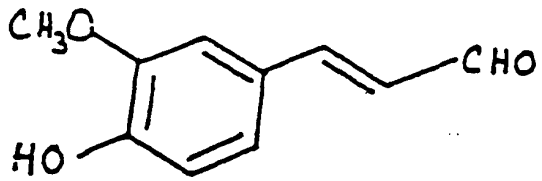
(2)

wherein R_1 represents-CHO, R_2 represents-OH or an organic substituent containing from 1 to 10 carbon atoms, R_3 represents a methoxy group or organic substituent containing from 1 to 10 carbon atoms, and R_4 represents a hydrogen or an organic substituent containing from 1 to 10 carbon atoms. Of particular interest are flavonoid aldehydes, particularly aromatic aldehydes. Examples of aromatic aldehydes of use in the present invention are cinnamic aldehyde ((3) below):

(3)

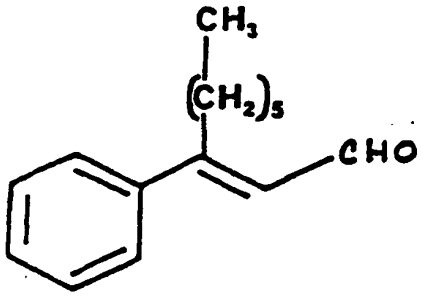


and coniferyl aldehyde ((4) below):



(4)

Other compounds of interest include analogs of the compound of formula (1) such as compounds substituted at the alpha position with an alkyl, such as a hexyl group, or a branched alkyl group such as an amyl group. Generally the group at the alpha position is from C-5 to C-10. Such compounds include α-hexyl cinnamaldehyde and α-amyl cinnamaldehyde. The chemical structure of α-hexyl cinnamic aldehyde (HCA) is shown in (5) below.



(5)

The Chemical Abstracts Service (CAS) name of HCA is 2-(phenylmethylene) octanal and the CAS Registry Number is [101-86-0]. The compound is also described by the chemical name of 2-hexyl-3-phenyl-2-propenal. The compounds's formula is $C_{15}H_{20}O$ and molecular weight is 216.3. HCA can be obtained from Firmenich; their product is composed principally of the (E)-cis isomer (93.8% maximum), and the (Z)-trans isomer (6% maximum). Among minor components is the self aldol condensation product of octanal (1-1.5% (Personal Communication, June Burkhardt, Firmenich, Plainsboro, New Jersey).

The compounds can be used either alone or in combination with other active or inactive substances and can be applied by sprayin¤, pouring, dipping, injecting, in the form of concentrated liquids, solutions, suspensions, powders and the like, containing such

concentration of the active compound as is most suited for a particular purpose at hand. They can be applied, for example, in the form of dilute solution, in a suitable solvent directly to the rhizosphere either as part of an irrigation schedule or as a separate application.

For use as a foliar spray, although the aldehyde can be formulated alone, it can be rendered substantive by including an emulsifier such as Tween 80. Other detergents which can be used include anionic detergents such as those described in U.S. Patent Application No. 4,978,686. Other compounds which can be used alone or in conjunction with detergents include saponins from any of a variety of sources, particularly saponin from *Yucca schidigera* or *Yucca valida*. Generally, detergents and other agents used in the formulation do not detract from the repellent properties of the flavonoid aldehydes but do increase the substantive properties of the formulation (*see* for example, U.S. Patent No. 4,477,361) and may improve the pesticide properties, including fungicide properties (see below). Additional components such as an aqueous preparation of a salt of a polyprotic acid such as sodium bicarbonate, sodium sulfate, sodium phosphate or sodium biphosphate can be included in the formulation, to increase the antifungal properties of the formulation. The resulting emulsion is diluted to an appropriate concentration for use.

In a preferred embodiment, the formulation includes α-hexyl cinnamic aldehyde, cinnamic aldehyde and/or coniferyl aldehyde in a formulation containing Tween 80 or saponin as an emulsifier and may include sodium bicarbonate. The preferred formulation for repelling flies, mosquitoes, fleas, ticks, lice, cockroaches, two-spotted spider mites, silverleaf white flies, aphids, leafhoppers, thrips and ants is 10-5000 ppm; for ticks, 100-2500 ppm. Generally, the total amount of aldehyde(s) present in the formulation is 2% or less. The formulations are effective without the use of antioxidants; particular aldehydes may have inherent antioxidant properties, for example, coniferyl aldehyde. Alcohols, such as glycols, including propylene glycol, are likewise not required for efficacy of the formulations and in fact may be harmful to the plant.

Stability of the formulation can be evaluated by a variety of methods, including accelerated tests in which a formulation of interest is exposed to elevated temperatures over a set time. Samples of the formulations are taken at regular intervals and analyzed chemically by methods known to those skilled in the art to determine the rate and nature of degradation. For example, HCA can be analyzed by Gas Liquid Chromatography (GLC), using a 30 meter non-polar polydimethylsiloxane capillary column (e.g. HP-1, Hewlett-Packard, or SPB-1, Supelco) and a flame-ionization detector (Personal Communication). Using helium as a

carrier gas (8 ml/min.) and a column temperature of approximately 240°C, the (E)-cis isomer (major component) has a retention time of approximately 6.0 minutes and the (Z)-trans isomer (minor component) has a retention time of approximately 6.3 minutes.

The most effective amount for compositions including compounds of formula (3) and/or formula (4) and/or formula (5) as well as the amount of other compounds of formula (1) which find use can be determined using protocols known to those of skill in the art for evaluating repellent efficacy of compounds. Examples of such protocols follow. These protocols also can be used to optimize each formulation for specific pathogens using any of the compounds encompassed by formula (1) or formula (5) as well as for specific applications to minimize plant phytotoxicity or skin sensitivity and other side effects for animals while maximizing the antipathogenic effect of the formulation.

In some instances, the efficacy of the formulation can be increased by adding one or more other components, *i.e.*, a compound other than a compound of formula (1) or (5), to the formulation where it is desirable to alter particular aspects of the formulation. As an example, it may be desirable for certain plant applications if there is an undesirable amount of phytotoxicity to decrease the phytotoxicity effect (phytotoxicity rating of 2 or less, with 1 or less preferred, *see* below) or to increase the repellent effect of the formulation, or both. It is preferable that the additional component(s) minimize any side effects to plants or animals while increasing the repellent effect of the formulation. Of particular interest is the use of a component(s) which is a synergist to increase repellency while minimizing any side effects as related to a particular formulation. By "synergist" is intended a component which, by virtue of its presence, increases the desired effect by more than an additive amount. The concentration of one or more of the other formulation ingredients can be modified while preserving or enhancing the desired repellent effect of the formulation. Of particular interest is the addition of components to a formulation to allow for a reduction in the concentration of one or more other ingredients in a given formulation while substantially maintaining efficacy of the formulation. Combination of such a component with other ingredients of the formulation can be accomplished in one or more steps at any suitable stage of mixing and/or application of the composition.

Preferred additional components include saponins, as they can be substituted for surfactants as emulsifying agents, and additionally on at least same plants have a growth promotant effect at the concentrations used. Generally, the use of saponin does not interfere with the repellant properties of the formulation. Saponins are a class of compounds, each

consisting of a sapogenin portion and a sugar moiety. The sapogenin may be a steroid or a triterpene and the sugar moiety may be glucose, galactose, a pentose, or a methylpentose. S. Budavari, ed., *The Merck Index*, 11th ed., Merck & Co., Inc., Rahway, N.J., 1990, p. 1328. The saponins for use in the present invention can be produced and/or isolated from various plant parts including fruit, leaf, seed and/or root, using means known in the art, from a variety of sources including the various plants known to produce them, ranging from yucca, quillaja, agave, tobacco, licorice, soybean, ginseng and asparagus to aloe woods. Saponins for use with the present invention are preferably non-toxic to humans and higher animals. Most preferably the saponin for use in the present invention is non-toxic food grade, the source being from yucca plants. Even more preferred are the saponins from *Yucca schidigera* or *Y. valida* and their equivalents. The saponins are generally prepared by a cold press extraction process and the resulting liquid extract used. The yucca fiber also can be used; it is typically sundried, mulled and sized by screening. Generally an effective amount of saponin is of the range 0.01 to 0.1% and most preferably about 0.01% v/v aqueous solution of 10^0 brix saponin extract.

A variety of structurally related saponins are known, the most variable structural feature being the glycosylation pattern. Saponins also may contain additional modifications, such as sarasaponins which are saponins with a steroid attached, and saponin structure can be modified by any number of enzymatic, chemical and/or mechanical means known in the art. Saponins from *Yucca schidigera* contain steroidal saponins with the major sapogenins being sarasapogenin and tigogenin. The sarasaponin yields on hydrolysis, sarasaspogenin (sarasaspogenin 5-beta, 20-betaF, 22-deltaF, 25-betaF; also known as spirostan-3-beta-01 and parigenin), glucose and galactose. The saraspogenin has a molecular formula of $C_{27}H_{44}O_3$. Nobel, Park S., *Agaves*, Oxford Univ. Press, New York, 1994. A variety of structurally related saponins are known, the most variable structural feature being the glycosylation pattern. S. Budavari, ed., *The Merck Index*, 11th ed., Merck & Co., Inc., Rahway, N.J., 1990, p. 1328. Saponins also may contain additional modifications, such as the sarasaponins which are saponins with a steroid attached, and saponin structure can be modified by any number of enzymatic, chemical and/or mechanical means known in the art. Generally, an effective amount of saponin is of the range of about 0.01 to 3% and most preferably about 0.25% v/v aqueous solution of 10^0 brix saponin extract. 10^0 brix is a terms of art in sugar chemistry. The brix degrees equals the percent by weight of sugar in the solution. Hawley,

ed., *The Condensed Chemical Dictionary*, 10th ed., Van Nostrand Reinhold, New York, 1981, p. 149.

For applications where the formulation is to be used to prepare the ground or other growth substrate for planting of host plants susceptible to particular pathogens, particularly where the growth substrate is already infested, the formulations of the subject invention can be added directly to the rhizosphere or the substrate or they can be bound to a solid support or encapsulated in a time release material to repel undesirable insects and other pests. Where a solid carrier is used, materials which can lead to oxidation of the active aldehydes should be avoided. Examples of delivery systems which can be used include starch-dextran, and the like. See Yuan *et al.*, *Fundamental and Applied Toxicology* (1993) 20: 83-87 for other examples of appropriate materials.

In addition to the specific compounds of the formulas (1), (2), (3), (4) and (5) above, precursors of any of these compounds that produce a compound of the formulas identified above upon action of a biological system on the precursors are considered to be equivalent to compounds of the invention. Thus application of precursor compounds to plant parts or tissues would be equivalent to the practice of the present invention. Biological conversion of precursor compounds into flavonoid aldehydes is described in, for example, U.S. Patent Application No. 5,149,715 and references cited therein. *See* also Casey and Dobb *Enzyme Microb. Technol.* (1992) 14: 739-747.

The method of the present invention is carried out by introducing onto a surface of interest a sufficient amount of an repellent agent to repel the insect or other pest. Alternatively, where the surface of interest is skin, fur, hair, clothing and the like, the application can be by way of contacting the surface of interest with a formulation that has been rendered substantive for the surface of interest so that a repellant amount of the formulation remains on the surface so treated and is released at a rate sufficient to repel susceptible insect or other pest. A formulation containing the repellent agent generally is introduced by topical application to a surface. For example, the formulation is sprayed on, as a wet or dry formulation, the surface and/or underside as applicable to the surface of interest. Among the formulations suitable for application are sprays, sticks, and repellent oils or ointments. In some instances, the surface of interest can be impregnated with the repellent formulation by absorption into the surface. Alternately, the formulation can be applied wet or dry to the rhizosphere where it can vaporize in the vicinity of the roots and associated pathogenic organisms which colonize the roots at a rate sufficient to repel a susceptible insect

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or pest. In some instances, air can be introduced into the rhizosphere to increase the vaporization process. To prevent an ingress of insects into an area, the compositions of the invention can be applied to surfaces within and/or surrounding the area, for example, the compositions can be applied to doors, windows and other openings of a building and/or to surfaces that surround these openings.

Where the surface of interest is a plant or plant part, the presence of the repellent agent can be a result of topical application; for example, the compositions can be aerially applied to crops, or it can be by elaboration from the host plant as a result of genetic modification of the host plant.

The aromatic and aliphatic aldehydes of the subject invention can be prepared by various synthetic methods known to those skilled in the art. For example, *see*, J. March, ed., Appendix B, *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 2nd Ed., McGraw-Hill, New York, 1977. Cinnamaldehyde can be prepared synthetically, for example, by oxidation of cinnamyl alcohol (Traynelis *et al., J. Am. Chem. Soc.* (1964) 86:298) or by condensation of styrene with formylmethylaniline (Brit. patent 504,125). The subject aldehydes also can be obtained by isolation from natural sources. For example, cinnamaldehyde can be isolated from woodrotting fungus, *Stereum subpileatum*. Birkinshaw *et al., Biochem. J.* (1957) 66:188, and α-hexyl cinnamic aldehyde (HCA) can be obtained from rice, or synthesized as described in USPN 5,055,621.

A preferred method for producing a desired component of the present formulations in a plant host is through recombinant DNA means, particularly by modifying the level of at least one compound of interest of the formula (1), (2), (3), (4), or (5) in plant tissues of interest through construction of transgenic plants using recombinant techniques known in the art. The methods involve transforming a plant cell of interest with an expression cassette functional in a plant cell comprising as operably linked components in the 5' to 3' direction of transcription, a transcriptional and translational initiation regulatory region, joined in reading frame 5' to a DNA sequence encoding and capable of modulating the production and/or required to produce the compound of interest, and translational and transcriptional termination regions. Expression of an enzyme required to produce the compound of interest provides for an increase in production of the compound as a result of altered concentrations of the enzymes involved in the compounds' biosynthesis. Of particular interest is the selective control of cinnamic and/or coniferyl aldehyde and/or HCA production in plant tissues such as leaves, roots, fruits and seeds. One or more compounds of the present

formulations can be produced by modulating the expression of one or more genes or a gene encoding or more enzymes or an enzyme pathway or cluster required to control the level of the compound of interest in a plant, plant part, plant cell, specific plant tissue and/or associated with a particular stage of plant growth.

The enzyme or enzymes can be in a biosynthetic pathway or a degradation pathway and the regulation will be up or down respectively; *i.e.*, to modulate expression of an indigenous or an endogenous plant gene an indigenous plant gene is one which is native to the genome of the host plant. An endogenous plant gene is one that is present in the genome of the plant host of interest, and may be an indigenous gene or a gene that is present as a result of infection of the plant (*e.g.*, a viral gene), or otherwise naturally incorporated into the plant genome. The host plant also can be modified by recombinant means or by traditional plant breeding methods to introduce one or more genes exogenous to the host plant which encode enzymes which control the level of the compound of interest and/or are in the synthetic pathway for one or more compounds of formula (1), (2), (3), (4) or (5). By "modulation of gene expression" is intended control of production of a gene product of interest at the level of transcription, translation and/or post translation. The level of the compound of interest is controlled by modulating the expression of one or more endogenous genes or transgenes encoding one or more enzymes required to synthesize the compound of interest.

Methods for modulating gene expression in plants are known in the art. Variation in growth conditions or exogenous application of compounds to a plant can affect gene expression. At the molecular level, gene expression depends substantially on the transcription, translation and termination control regions which regulate expression of a structural gene coding region. By exploiting the plant signals which regulate these control regions or by the direct recombinant manipulation of the control regions, expression of a gene encoding an enzyme required to control the level of cinnamic aldehyde, for example, can be modulated. For use in a transgene supplied exogenously to a plant host, the transgene will include control regions that are selected and designed to achieve the desired tissue and/or level and timing of gene expression. As appropriate, the control regions may be homologous (native) or non-homologous (non-native) to the gene of interest. By "homologous" it is meant that the control region(s) is from or substantially similar to a control region normally associated with the gene of interest. By "non-homologous" it is meant that the control region(s) originates from a different nucleotide source or sequence or is substantially different

from the control region(s) normally associated with the gene of interest. For example, if the enzyme coding sequence is non-homologous in source as compared to the control regions, in order to have expression of the gene in a plant cell of interest, transcriptional and translational initiation regulatory regions or promoters functional in these plant cells must be provided operably linked to the coding sequence. Transcription and translation initiation signals functional in plant cells include those from genes which are present in the plant host or other plant species, and direct constitutive or selective expression in a plant host.

Of particular interest are the gene control regions that selectively regulate structural gene expression in a plant, plant part, plant cell, specific plant tissue and/or are associated with a particular stage of plant growth. Preferred are those control regions, that are known in the art, and in particular, transcriptional control regions or promoters, that can be used to modulate the expression of a gene encoding an enzyme required to control the level of a compound of formula (1), (2), (3), (4) and/or (5) in a plant, plant part, plant cell, or specific plant tissue and/or are associated with a particular stage of plant growth. For example, promoters showing differential expression patterns in fruit are described in USPN 4,943,674 and USPN 5,175,095; seed in USPN 5,315,001; and in rapidly developing tissues and tender shoots in USPN 5,177,011.

For selective control of biosynthesis of cinnamic and/or coniferyl aldehyde and/or HCA in a plant tissue of interest, plant cells are transformed with an expression cassette comprising DNA encoding a structural gene for one or more enzymes required to synthesize cinnamic and/or coniferyl aldehyde and/or HCA and capable of increasing the amount of these aldehydes in the tissue of interest. Of particular interest are those genes encoding one or more enzymes capable of metabolizing a precursor compound required for the biosynthesis of cinnamic and/or coniferyl aldehyde and/or HCA from substrates normally found in a plant cell, more particularly the transgenic expression of at least one compound of the formula (1), (2), (3), (4), or (5).

DNA constructs for expressing a gene of interest can be prepared which provide for integration of the expression cassette into the genome of a plant host. Integration can be accomplished using transformation systems known in the art such as *Agrobacterium*, electroporation or high-velocity microparticle-mediated transformation. Depending upon the application, saponin or one of the other compounds of interest can be preferentially expressed in a tissue of interest and/or a particular organelle. Tissue specificity is accomplished by the use of transcriptional regulatory regions having the desired expression profile. Translocation

of the enzyme to a particular organelle is accomplished by the use of an appropriate translocation peptide. Methods for tissue and organelle specific expression of DNA constructs have been described are known in the art.

To verify regulation and expression of the gene of interest, various techniques exist for determining whether the desired DNA sequences present in the plant cell are integrated into the genome and are being transcribed. Techniques such as the Northern blot can be employed for detecting messenger RNA which codes for the desired enzyme. Expression can further be detected by assaying for enzyme activity or immunoassay for the protein product. Most preferably the level of the compound of interest present in a plant host is measured using methods known in the art. A desired phenotype, for example, is increased HCA content in a plant tissue of interest as measured by expression of the gene of interest and/or the level of HCA present in the plant host as compared to a control plant.

For introduction of one or more compounds of the present formulations to the target organism, a plant host expressing a gene encoding an enzyme required to control the level of the compound of interest results in the exposure of a target organism to at least one component of the repellent formulation. At least one component of the repellent formulation can be expressed by the plant host and optionally other components of the repellent formulation are exogenously applied to the plant host so that the combination elicits the desired repellent effect.

Transgenic plants having an increased ability to accumulate flavonoid aldehydes such as cinnamaldehyde and coniferyl aldehyde and HCA to provide self-protection against plant pests or be used as a natural source of flavonoid aldehydes for extraction and subsequent use as a repellant can be prepared.

Accumulation of flavonoid aldehydes can be achieved by downregulating the expression of specific plant genes that encode enzymes which either cause further metabolism of the desired aldehydes or divert metabolic intermediates away from the desired aldehydes. In the case of cinnamaldehyde, for example, this involves downregulating the expression of cinnamate 4-hydroxylase (CA4H) and cinnamic alcohol dehydrogenase (CAD). CA4H ordinarily diverts some cinnamic acid away from cinnamaldehyde to produce *p*-coumaric acid, itself a metabolic intermediate. Reducing CA4H activity alone is not sufficient to cause accumulation of cinnamaldehyde because CAD can rapidly convert cinnamaldehyde to cinnamyl alcohol, which then becomes incorporated into lignin or accumulates as glycosides. Simultaneously reducing both CA4H and CAD activities results in increased metabolic flux

from cinnamic acid into cinnamaldehyde and decreased conversion of cinnamaldehyde into cinnamyl alcohol. Some cinnamaldehyde becomes incorporated into lignin but cinnamaldehyde (either free or as glycosides) also accumulates to above-normal levels, particularly at times when the biosynthesis of cinnamic acid is elevated. This occurs when the level of phenylalanine ammonia lyase (PAL; the first and rate-limiting step in general phenylpropanoid metabolism, Hahlbrock and Scheel (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40:347-369) activity is high, a situation that naturally occurs in plants in response to a wide range of stimuli including invasion by fungal pathogens and mechanical damage associated with wounding and insect feeding.

Inhibiting CAD activity in transgenic plants has been proposed as a method of reducing lignin synthesis in plants and thereby improving the digestibility of fodder crops (WO 93/05159). These experiments suggested that lignin biosynthesis had been altered qualitatively, but not necessarily quantitatively, but did not demonstrate or appreciate the desirability of accumulating cinnamaldehyde as a method of increasing insect and other pest repellancy.

A number of plant CA4H and CAD genes have been cloned and their sequences are available from GenBank. Portions of these genes that include nucleotide sequences that are conserved between different plant species can be used directly in a plant expression vector (antisense or sense orientation) to suppress the expression of the corresponding endogenous genes (*e.g.*, Pear, *et al.*, *The Plant Cell Antisense Res. and Develop.* (1993) 3:181-190, Napoli, *et al.*, *The Plant Cell* (1990) 2:279-289. More preferably, these conserved gene sequences are used to isolate CA4H and CAD cDNA clones from a cDNA library of the plant species that is to be modified. The resulting cDNA clones, or portions thereof, are then introduced into a plant expression vector (antisense or sense) and used to transform the plant(s) of interest. DNA constructs according to the invention preferably comprise a sequence of at least 50 bases which is homologous to the endogenous CA4H or CAD genes.

A recombinant DNA molecule can be produced by operatively linking a vector to a useful DNA segment to form a plasmid that can be used for plant transformation. A vector capable of directing the expression of RNA from a cloned portion of a gene is referred to herein as an "expression vector." Such expression vectors contain expression control elements including a promoter. Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the Ti plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, *Methods in Enzymology* (1987)

153:253-277. A common promoter that is used to provide strong constitutive expression of an introduced gene is the cauliflower mosaic virus (CaMV) 35 S promoter (available from Pharmacia, Piscataway, NJ). Either constitutive promoters (such as CaMV 35S) or inducible or developmentally regulated promoters (such as the promoter from a PAL gene or the endogenous CA4H or CAD genes) can be used. Use of a constitutive promoter will tend to affect functions in all parts of the plant, while use of an inducible or developmentally regulated promoter has the advantage that the antisense or sense RNA is only produced in the tissue and under the conditions it is required. The use of developmentally regulated promoters is preferred in the use of this invention because the down-regulation of phenylpropanoid biosynthesis is known to be capable of producing undesirable side-effects on the development of transgenic plants containing a heterologous PAL gene (Elkind, Y. *et al., 1990) Proc. Nat. Acad. Sci.* (1990) 87:9057-9061.

A number of different transformation methods are available for the routine transformation of a wide range of plant species. One method that is particularly efficient for the transfer of DNA into dicotyledonous plants involves the use of *Agrobacterium.* In this method the gene of interest is inserted between the borders of the T-DNA region that have been spliced into a small recombinant plasmid with a selectable marker gene (for example encoding neomycin phosphotransferase II or phosphinothricin acetyltransferase). The recombinant plasmid is then introduced into an *Agrobacterium* host by transformation or triparental mating. The *Agrobacterium* strain carrying the gene(s) of interest is then used to transform plant tissue by co-culturing the bacteria with an appropriate plant tissue (*e.g.*, leaf disc). Transformed cells are selected in tissue culture using the appropriate selection agent and plants are then regenerated *(see* Horsch, R. B. *et al., Science* (1985) 227:1229-1231. Other methods that have been used in the transformation of plant cells, and in particular the more recalcitrant crop plants, include biolistics and electroporation (for detailed protocols, *see Sanford, et al.*, (1993) *Methods in Enzymology* 217:483-509; and Potter, (1993) *Methods in Enzymology* 217:461-478.

Once transgenic plants have been produced, conventional enzyme assays for CA4H and CAD are used to determine the level of suppression of enzyme activity achieved in different transformants. It is likely that only a small fraction of the transformants produced will have a sufficiently low residual enzyme activity to cause the accumulation of flavonoid aldehydes without also producing some undesirable side-effects on plant development. For this reason, a preferred method of producing the desired transformants with both CA4H and

CAD suppressed is to introduce the two genes separately into different transformants and then combine them by standard sexual crosses. This permits a larger number of combinations of level of gene suppression to be evaluated at the same time.

An alternative to overproducing flavonoid aldehydes in transgenic plants is to use the plant genes to confer on a microbial host the capability of synthesizing specific flavonoid aldehydes. The resulting microbes can be used either to produce the flavonoid aldehydes in a fermentation system or as a natural delivery system of the flavonoid aldehydes in viable or non-viable microbial preparations. Yeasts, especially *Saachoromyces cerevisiae*, are preferred organisms for this purpose because they have already been engineered for high-level expression of PAL (Faulkener *et al.* (1994) *Gene* 143:13020) and a plant cinnamate 4-hydroxylase has been shown to function in yeast (Urban *et al.* (1994) *Eur. J. Biochem.* 222:843-850).

The expression of PAL introduces the capability to produce cinnammic acid from phenylalanine. Two additional enzymic steps are required to produce cinnamaldehyde from phenylalanine. In plants, these steps are catalyzed by the enzymes cinnamate:CoA ligase (CL) and cinnamoylCoA reductase (CCoAR), but as 4-coumarateCoA ligase (4CL) can also use cinnamic acid as substance (Knobloch, and Hahlbrock (1977) *Arch. Biochem. Biophys.* 184:237-248), 4CL can be used instead of CL. More than 20 cloned PAL genes and more than 6 4CL genes have been described in sufficient detail (GenBank) to facilitate their use in practicing the current invention. A gene for a CCoAR is obtained by applying standard gene cloning techniques to isolate a cDNA clone using as a probe sequence derived from the amino acid sequence of the N-terminus, or peptide fragments, of the purified protein. CCoAR has been purified and partially characterized from soybean cultures (Wengenmayer *et al.* (1976) *Eur. J. Biochem*, 65:529-536; Luderitz and Grisebach (1981) *Eur. J. Biochem*, 119:115-124), spruce cambial sap (Luderitz and Grisebach, *supra*), poplar xylem (Sarni *et al.* (1984) *Eur. J. Biochem*, 139:259-265) and differentiating xylem of *Eucalyptus gunnii* (Goffner *et al.* (1994) *Plant Physiol.* 106:625-632). The preferred method of purification is that of Goffner *et al.* (*supra*) because it results in a single protein band on SDS-polyacrylamide gels that an be used for protein sequencing.

The cloned genes are introduced into standard expression vectors and used to transform a microbial host, preferably yeast, by standard transformation techniques such as electroporation (Becker and Guarante (1991) *Methods in Enzymol.* 194:182-187). Standard enzyme assays are used to confirm the functional expression of the engineered genes and

assays for flavonoid aldehydes are used to select strains with maximal production. Because flavonoid aldehydes have antimicrobial properties it is preferred to use expression vectors that will cause expression of the introduced genes only late in the growth cycle or in response to a chemical inducer. It may also be desirable to grow the engineered microbial host in an immobilized whole cell reactor (*e.g.*, Evans *et al.* (1987) *Biotechnology and Bioengineering* 30:1067-1072) to prevent the aldehydes from accumulating in the culture medium.

The target insects and other pests include those which are vectors for disease organisms such as fungi which colonize a surface of a part of a plant which is an elicitor for the fungus. By elicitor is intended that the plant secretes nutrients required by the fungus. Examples of fungi and the plant parts which they colonize are as follows. Black spot on fruit; *Fusarium sp.* on flowers roots and leaves; and *Fusarium spp.* and *Aspergillus* on roots and leaves. *Fusarium* causes vascular wilts of annual vegetables and flowers, herbaceous perennial ornamentals, plantation crops and the mimosa tree. Different plants are attacked by special forms or races of the fungus. *Verticulum* (*V. albo-atrium* and *V. dahlise*) cause vascular wilts and colonize roots, flowers and leaves. In addition the following also constitute target organisms: *Phragmidium spp*; *Diplocaopan rosae*; *Sphaerotheca tannosa*; *Oibiapsis sicula*; *Phytophthora taraesitica*; *Phytophthora infestans*, *Puccinia spp*; *Alternaria spp*; *Susaiun spp*; *Botrytis cinera*; *Sclerotinia homoeocarca; Tricophyton mentagrophpytes*; Dutch Elm disease (*Ceratocystis ulmi*) and oak wilt (*C. fagacearum*). *Ceratocystis* causes vascular wilts, mainly of trees. Also included are blue-green algae (Cyanobacteria). The vectors for these diseases which can be repelled by the subject formulations include beetles and wasps. Target organisms also include insects which damage the plants which they colonize, particularly those of the orders *Orthoptera*; *Thysanoptera* which includes water weevil and thrips; and *Homoptera* which include aphids such as root aphid and leaf aphid, leafhoppers, white flies, mealy bugs, thrips, cicadas, caterpillar, such as velvet bean caterpillar, codling moth, leaf roller, and scale insects. Other target organisms include arachnids (particularly spider mites), flies *(Musca domestica)*, cockroaches, gastropods, moths, and bed bugs (*Cimex lectularis*) and its close relatives (poultry bug (*Haematosiphon indorus* Duges), the European pigeon bug (*Cimex columbarius* Jerjus), and the swallow bug (*Oeciains vicarius* Hrovath)).

Also of particular interest is prevention of phylloxera infestation in grapes by repelling the phylloxera. For this application, it is necessary to deliver the formulation to the roots of the plants which are the usual habit for phylloxera. When used in a solid form or

microencapsulated, the dosage used is typically on the order of 1% to 35% on a w/w basis, the maximum loading to be determined as a function of shell material selected. Analytical chemical techniques are used to determine and optimize rate of release. For qualitative purposes, GC techniques can be used to determine the amount of aldehyde released. The samples of encapsulated (pelletized) product are mixed with the soil types selected and sampled at different time periods to measure release. Alternatively, the volatile gases released from the formulation can also be analyzed. For measuring the activity of foliar and drip irrigation applications the stability of the formulations over time can also be evaluated using the GC methodology using methods known to those skilled in the art. Methanol or alcohol extractions of the formulations also can be prepared for HPLC analysis. The preferred method of repelling phylloxera and other root dwelling pests, however, is to provide for a systemic response to, for example, a foliar application of the formulation which is then translocated to the root. The timing of such applications will need to be determined empirically for particular plants as the flow of water from the leaves to the roots is required for translocation. Generally, such flow is greatest at cooler temperatures *e.g.* during the evening hours, at night, or in the early morning hours, and pre or post fruit or vegetable development.

The subject formulations, in particular those containing HCA, are also useful for treating: grape to repel pests such as thrips, nematodes, and leaf roller; roses to repel thrips and melon aphids; cattle to repel soft ticks; humans to repel mosquitos; apple to repel codling moth; animals to repel fleas; cockroach habitats to prevent or eliminate cockroach infestation; and corn to repel root aphid.

In addition to treating a host plant, seeds can also be treated using the subject formulations to repel insects and other pests which attack the seeds and/or which act as vectors for disease organisms. The seeds can be dusted with a powder preparation (*see* U.S. Patent Application No. 4,978,686 for examples of inorganic materials to which the formulations can be adsorbed) or admixed in a plant substrate such as vermiculite. Seedlings grown under sterile conditions from treated seeds are free of susceptible fungi and insects. Additionally, seedlings also can be treated with the subject formulations. In some instances it may be necessary to adjust the treatment formulation so as to reduce any phytotoxicity associated with the treatment as tender young shoots are more likely to exhibit phytotoxicity symptoms. The treatment formulations are also useful for controlling the time of pollination of flowering plants. For example, to prevent or delay pollination the formulations are

applied in an amount sufficient to repel bees and other pollinating insects. By adjusting the residuality of the formulation, one can control the length of time during which pollination is inhibited. On the other hand, for plants whether cross-pollination is required for fertilization, application of the formulation during this period should be avoided if the pollinating insect is repelled by the formulation.

In order to determine the susceptibility of particular insects to repellency by the claimed compositions, *in vitro* and *in vivo* tests which compare the behavior of the target pest towards, for example, approaching a "bait" food in the presence and absence of the test formulation are used. The effectiveness of the formulation over time can be evaluated by extending the time period of observation until few of the test insects (less than about 50%) are repelled from the vicinity of the bait. For pathogen vector insects, a 90% or greater repellency is usually in order. For the common nuisance pest, reduction in the magnitude of 80% is suitable (*e.g.*, in garden and food areas). The formulations also need to be evaluated for phytotoxicity for use on plants and for dermal sensitivity, particularly for use on skin and/or clothing of humans; contact dermatitis and olfactory sensitivity are monitored using tests for dermal sensitivity known to those of skill in the art. Likewise, phytoxicity testing can be done using methods known to those of skill in the art. Phytotoxicity can be rated as follows in order of increasing severity of toxicity: 0-plants without any symptoms; 1-very slightly browning of hypocotyl (no other symptoms); 2-some wilting of plant, dying of lower leaves, some browning of vascular system; 3-wilting of entire plant, leaves dying, hypocotyl with external and internal symptoms; 4-necrosis of stem, plant dying. It is preferable that the formulation used have a phytotoxicity rating of 2 or less, more preferably 1 or less.

The components of a formulation to be used for a particular application can be determined by evaluating first the concentration range over which a given component has no activity to where it provides maximum activity (a dose response curve) and then evaluating this component separately and in combination with other components of interest for a given formulation. The repellent and/or phytotoxic and/or dermal effects of a particular formulation on a given insect or other pest and the host is then measured for each formula and component with or without a serial diluent of any additional component of interest. Optimal dose-ranges are calculated *in vitro* and *in vivo* using techniques known to those of ordinary skill in the art. Formulations are identified which provide: repellency of 90%, and/or a phytotoxicity rating of 2 or less for plants, with 1 or less being optimum, and substantially free of contact dermatitis for animals and fowl.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

The following products were used in the example protocols set forth below: (1) cinnamic aldehyde from Spectrum Chemical Co., New Jersey, USA; (2) coniferyl aldehyde from ADIN Chemical Co., VF; (3) sodium bicarbonate and Tween 80 from Spectrum Chemical Co., New Jersey, USA.

Example 1

Flies *(Musca domestica)*

The purpose of this experiment is to evaluate the repellency activity of cinnamic aldehyde and α-hexyl cinnamic aldehyde against flies *(Musca domestica)*. Twenty 2-3 day old female flies are released in a 62 x 62 x 34 cm cage with 325 mesh roof screening to permit air circulation (Carolina Biological Supplies). Bait made of sweet milk (Carnation) (90%) plus glucose (10%) and a dye (bromophenol blue 0.01%) with 1 ml of formulation is placed in a 3.5 cm petri dish and set inside a pine cage (Carolina Biological Supplies) with a 1 cm inch diameter hole drilled through the top to permit access to the cage containing the bait. A 3.5 cm petri dish with 5 ml H_2O is placed in the cage for water. After 24 hours, flies are removed and crushed on filter paper to check for the presence of dye which would indicate feeding activity. Entry of more than 10% of the flies is taken as an indication of lack of formula repellent activity.

Example 2

Cockroaches *(Blatella germanic)*

The aim of this experiment is to evaluate the repellancy activity of cinnamic aldehyde formula against cockroaches *(Blatella germanic)*. Fifty nymphs and adults 1.5 cm to 3.5 cm in length are released in a 62 x 62 x 34 cm cage with 325 mesh roof screening to permit air circulation. The inner surface of the cage walls from 5 cm to 10 cm from the floor are treated with a mixture of mineral oil and petroleum jelly (2:3) to prevent escape of the cockroaches. The cockroaches are fed on dog chow (Purina), milk powder and water for 48 hours for acclimatization. Two Whatman filter papers "C" (4 x 4 cm) are folded twice, and stapled and wet with 1 ml of formula each. Filter papers are allowed to air dry. After

air drying, a filter paper is placed inside of one of two 4 cm x 4 cm x 4 cm cubes, each with a single 0.75 cm door at floor (base) level for entry. The two shelter boxes are placed on the bottom floor of the cage 14 cm apart. After twenty hours, the shelters are removed and the number of shelter cockroaches are removed and counted. An entry of more than 10% of cockroaches into the shelter boxes is regarded as an indication of loss of formula repellency.

Example 3

Aphid *(Aphid fabae)*

The purpose of this experiment is to determine the repellancy activity of a cinnamic aldehyde formulation against black bean aphids. Sugar beet plants *(Beta vulgaris)* are grown in 7.5 mm pots in potting soil in a greenhouse. When plants reach the three leaf stage, eight plants are selected at random. In separate trials, four plants are treated with: cinnamic aldehyde at 50 ppm; 50 ppm cinnamic aldehyde formula ($NaHCO_3$ + Tween 80); $NaHCO_3$; Tween 80; and formula blank. Treatment is a foliar application of 5 ml of material sprayed as fine mist by a hand sprayer (Gilmour). Four plants are untreated and one sprayed with water only. The treated and untreated plants are placed in two rows, A or B, treated or untreated, respectively, in a 60 x 60 x 30 cm box cage with a 325 mesh screen roof permitting air circulation. At 4, 8 and 24 hours, the number of aphids on treated and untreated plants, rows A and B, are counted and recorded.

Example 4

Silverleaf White Fly *(Tetranychus urticae)*

The purpose of this experiment is to determine the repellency of cinnamic aldehyde against silver leaf white fly. In a greenhouse, cotton plants are grown in 7.5 mm pots in potting soil. When plants reach the three leaf stage, eight plants are selected at random. In separate trials, four plants are treated with: cinnamic aldehyde at 50 ppm; 50 ppm cinnamic aldehyde formula ($NaHCO_3$ + Tween 80); $NaHCO_3$; Tween 80; and formula blank. Treatment is a foliar application of 5 ml of material sprayed as fine mist by a hand sprayer (Gilmour). Four untreated plants receive a foliar spraying of 5 ml water. The treated and untreated plants are placed in two rows, A or B, treated and untreated, respectively, in a 60x 60 x 30 cm cage with a 325 mesh wire screen roof allowing air circulation. At 4, 8 and 24 hours, the number of silverleaf white flies are counted for presence on plants in rows A and

B (treated and untreated). At 48 hours, the number of eggs on plants in each row are counted and recorded.

Example 5

Leafhoppers *(Cicadellidae)*

The purpose of this experiment is to determine the repellency of cinnamic aldehyde against leafhoppers. In a greenhouse, cotton plants are grown in 7.5 mm pots in potting soil. When plants reach the three leaf stage, eight plants are selected at random. In separate trials, four plants are treated with: cinnamic aldehyde at 50 ppm; 50 ppm cinnamic aldehyde formula ($NaHCO_3$ + Tween 80); $NaHCO_3$; Tween 80; and formula blank. Treatment is a foliar application of 5 ml of material sprayed as fine mist by a hand sprayer (Gilmour). Four untreated plants receive a foliar spray of 5 ml of H_2O. The treated and untreated plants are placed in two rows, A or B, treated and untreated, respectively, in a 60 x 60 x 30 cm box cage with a 325 mesh wire screen roof allowing air circulation. At 4, 8 and 24 hours, the number of leafhoppers are counted for presence on plants in rows A and B (treated and untreated). At 48 hours, the number of eggs on plants in each of the rows is counted and recorded.

Example 6

Thrips *(Thysanoptera)*

The purpose of this experiment is to determine the repellency of cinnamic aldehyde against thrips. In a greenhouse, tomato plants are grown in 7.5 mm pots in potting soil. When plants reach the three leaf stage, eight plants are selected at random. In separate trials, four plants are treated with: cinnamic aldehyde at 50 ppm; 50 ppm cinnamic aldehyde formula ($NaHCO_3$ + Tween 80); $NaHCO_3$; Tween 80; and formula blank. Treatment is a foliar application of 5 ml of material sprayed as fine mist by a hand sprayer (Gilmour). Four untreated plants receive a foliar spray of 5 ml of water. The treated and untreated plants are placed in two rows, A and B, treated or untreated, respectively, in a 60 x 60 x 30 cm cage with a 325 mesh wire screen roof allowing air circulation. At 4, 8 and 24 hours, the number of thrips are counted for presence on plants in rows A and B (treated and untreated). At 48 hours, the number of eggs (in leaf slits) on plants in each row are counted and recorded.

Example 7

Twospotted Spider Mite *(Tetranychus urticae)*

The aim of this experiment is to evaluate the repellency of cinnamic aldehyde on twospotted spider mites. Cotton plants are grown in 7.5 mm pots in potting soil in a greenhouse. When plants reach the three leaf stage, eight plants are selected at random. In separate trials, four plants are treated with: cinnamic aldehyde at 50 ppm; 50 ppm cinnamic aldehyde formula ($NaHCO_3$ + Tween 80); $NaHCO_3$; Tween 80; and formula blank. Treatment is a foliar application of 5 ml of material sprayed as fine mist by a hand sprayer (Gilmour). Four untreated plants each receive a foliar spray of 5 ml of water. The treated and untreated plants are placed in two rows, A or B, treated or untreated, respectively, in a 60 x 60 x 30 cm cage with a 325 mesh wire screen roof allowing air circulation. At 4, 8 and 24 hours, the number of spider mites are counted for presence on plants in rows A and B. At 48 hours, the number of eggs on plants in each row are counted and recorded.

Example 8

Mosquito *(Aedes aegypti)*

Repellency Test Procedure *In Vitro*

The purpose of this experiment is to evaluate the repellency of cinnamic aldehyde against mosquitos. Twenty unblooded adult female mosquitoes approximately 4 days of age are introduced into test chambers. Four ml of a test formulation is pipetted onto a 16 cm #2 Whatman filter paper circle and air dried. The treated filter paper is placed on the vent intake chamber. CO_2 is bubbled through water at the vent intake end of a wind tunnel olfactometer chamber; the lowest rpm fan setting is used. The trap chamber is opened for 5 minutes, then closed and the number of mosquitoes counted and recorded. DEET at 23% is used as a positive control.

Repellency Test Procedure, Field Trial

The purpose of this experiment is to bioassay the activity of cinnamic aldehyde as a mosquito repellent. Two circles 18 cm in diameter and two 16 cm in diameter were cut from 1 mm mesh nylon mosquito cage bolt of screen material. The treatment circle (16 cm) was soaked in 1 ml formulation: cinnamic aldehyde (2%) in 2% Tween 80 and 6% $NaHCO_3$, then allowed to air dry for 2 hours. Ten unfed female *Aedes aegypti* mosquitoes (5-7 days

old) from the Kearney Agriculture Center, Mosquito Control Research Laboratory, were introduced into each of two Kearney (Fischer) one pint mosquito cartons (control and treatment cartons). Each carton was covered with one of the untreated 18 cm circle mesh screens and sealed with a rim from the pint carton, the lid section having been removed. An adult male volunteer placed the treated 16 mm circle on one of his legs (which had been washed and rinsed with soap and water) and the untreated 16 mm circle on the other leg (washed and rinsed with soap and water). The one pint mosquito cartons were put in flush contact with the mesh screen side to the leg screen patches for 5 minutes. Mosquitoes did not come in direct contact with compound. After 5 minutes, the number of blooded/well-gorged insects out of 10 were counted. The results are shown in Table I, below. Out of a total of thirty insects evaluated, only two were not repelled by the cinnamic aldehyde formulation, as compared to 19 in the control (untreated) group.

Table I

Mosquito Repellancy

(# of blooded/well-gorged insects/10 insects)

	Trial 1	Trial 2	Trial 3	Sum
Cinnamic aldehyde formulation[1]	0/10	2/10	0/10	2/30
Control (untreated)	5/10	7/10	7/10	19/30

[1] Cinnamic aldehyde (2%) with 2% tween 80, and 6% $NaHCO_3$ in H_2O.

A protocol similar to that described above is used to test α-hexyl cinnamic aldehyde.

Example 9

Lepidopteran ovipositional Repellency

The purpose of this experiment is to determine the repellency of cinnamic aldehyde against Beet Armyworm adult moths. An apparatus is built that forces an airstream over treated and non-treated potted plants in a flight cage. Five tomato plants at the three leaf stage are treated with 5 ml of various concentrations of

chemical formula and components and then placed in the cage. Five tomato plants are sprayed with 5 ml of H_2O as control plants and then placed in the cage. Forty egg laying ready Beet Armyworm *(Spodoptera exigna)* adults are released in the cage. The exhaust fan on the apparatus is turned on and a low velocity linear flow of air is allowed to flow through the cage as plumelets of air evaporating chemical formula. After 24 hours, oviposition is determined on treated plants, non-treated plants, and cage walls.

Example 10

Phylloxera - Vapor Test for Repellency

The purpose of this experiment is to evaluate the vapor repellency of cinnamic aldehyde to phylloxera. Root pieces of grape stock with viable phylloxera eggs (n-30) is placed in 50 x 9 mm dishes that have been treated on inner surfaces with 400 ml of known product concentration. The chemical is not placed directly on the root, so that absorption or metabolism by the root is not a factor. The dishes are shut and sealed with tape. After 7 days, the dishes are opened, and a determination is made as to whether insects are able to establish on the roots and develop, or whether the newly hatched insects avoid the roots and die.

Example 11

Overproduction of Flavonoid

Aldehydes in Transgenic Plants

Twenty μg of polyA RNA is prepared and cDNA synthesized. Part of this is cloned into lambda-ZAP II vector (a commercially available cloning vector). At least 500,000 recombinants are screened using an oligonucleotide probe designed from conserved sequences of cloned CA4H and CAD genes obtained from GenBank, or designed from peptide sequence of purified protein from the intended host plant. Strongly hybridizing clones are selected and used to rescreen the cDNA library. The resulting clones are sequenced to enable the introduction of appropriate gene sequences into a plant expression cassette in either antisense or sense orientation. The antisense and sense constructs are introduced into *Agrobacterium tumefaciens* LBA4404 by direct transformation following published procedures. Tobacco (*N. tabacum*, variety Samsun) leaf discs are transformed using well estabished published

procedures (Horsch *et al.* (1985) *Science* 227:1229-1231. Plants containing either CA4H or CAD constructs are identified by PCR and selected for further analysis.

Plant material from both transformed and untransformed control plants is used for determinations of CA4H and CAD enzyme activity using well established published assays. Plants in which the activity of CA4H or CAD has been reduced to less than 20% of that seen in control plants are selected for further analysis. Selected plants with low CA4H activity are crossed with plants with low CAD activity and progeny inheriting both gene constructs are selected by PCR. Plants with suppressed CA4H and suppressed CAD activity are analyzed for flavonoid aldehyde production using standard published procedures. Those plants that produce flavonoid aldehydes are then tested for efficacy of repelling insects or other pests using any appropriate example, *e.g.* Example 3 to test transgenic cotton plants for their capacity to repel aphids.

EXAMPLE 12

Production of Flavonoid Aldehydes in Microbial Systems

A cDNA library is generated using RNA extracted from six week old tobacco stems. 20μg of polyA RNA is prepared and cDNA synthesized. Part of this is cloned into lambda-ZAP II vector (a commercially available cloning vector). At least 500,000 recombinants are screened using an oligonucleotide probe designed from peptide sequence sequences of CCoAr protein purified from six week old tobacco stem tissue using the protocol of Goffner *et al.* (1994) *Plant Physiol.* 106:625. Strongly hybridizing clones are selected and used to rescreen the cDNA library. The resulting clones are sequenced to enable the identification of full-length cDNA inserts and the introduction of appropriate CCoAR gene sequences into yeast expression vector pMTL8110 (Faulkner *et al.* (1994) *Gene* 143:13-20. The coding sequences for *Rhodosporidium toruloides* phenylalanine ammonia lyase (PAL; GenBank locus RHDPAL) and a parsley 4-coumarate:CoAl ligase (4CL; GenBank locus PC4CL1AA) are similarly introduced into equivalent yeast expression vectors. The PAL,4CL and CCoAR constructs are used to transform *Saccharomyces cerevisiae* strains by electroporation using established published procedures (Becker, and Guarente, *Methods in Enzymology* 194:182-187, 1991; Simon (1993) *Methods in Enzymol* 217:478-483. Transformants are selected on minimal medium lacking leucine.

Transformant strains carrying all three gene constructs are identified by PCR and selecter for further analysis.

Extracts from both transformed and untransformed control strains are used for determinations of PAL, 4CL and CCoAR enzyme activities using well established published assays. Strains in which the activity of PAL, 4CL and CCoAR is significantly greater than the background activity detected in control strains are selected for further analysis. Selected strains are analyzed for flavonoid aldehyde production using standard published procedures and those producing significant amounts of cinnamaldehyde are selected for optimization of fermentation conditions. The resulting products are then tested for their efficacy in repelling insects and other tests using any of the described methods.

Example 13

HCA Activity as Insect Repellant

The purpose of this experiment is to determine whether α-hexyl cinnamic aldehyde is an effective insect repellant. Following negative skin-irritation tests on rabbits at the FDA, HCA was evaluated on the skin of 2 to 4 male human subjects. One ml of the compound was rubbed over one forearm. A glove was worn to protect the untreated hand while the treated forearm was exposed in a cage containing a high number (2,000-4,000) of unfed mosquitoes for 3 minutes at intervals of approximately 30 minutes until two bites were received (two bites in one test period or one bite in each of two consecutive test periods). The time interval between application and when two bites were received was defined as the "protection time." Against the yellow fever mosquito (*Aedes aegypti* (L)), HCA was rated as 3 (121-180 minutes duration). Against the malaria mosquito (*Anopheles quadrimaculatus* Say), HCA was rated as 2 (31-60 minutes).

HCA was also evaluated on treated cloth against the yellow fever mosquito. In these tests, women's mercerized-cotton stockings were used. A measured section above the ankle was impregnated with HCA at a rate equivalent to 3.3 g/ft2. The stocking was spread on a rack to dry and then hung indoors on a line. The first tests were done 24 hours after treatment. The stocking was drawn over the arm, with the treated portion midway on the forearm. The untreated hand was protected with a glove and the stocking-covered arm was exposed for one minute in a test cage. If 5

bites were received, the treatment was considered ineffective. If less than 5 bites were received, the exposures were continued daily until the 14th day and at weekly or biweekly intervals thereafter. HCA received a grade of 4 in this test (effective for more than 10 days), the same value as reported for DEET.

These examples demonstrate that the subject cinnamic aldehyde and α-hexyl cinnamic aldehyde formulations are effective repellents against mosquitos.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now having been fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

1. A composition for repelling pests, said repellant composition comprising at least one flavonoid aldehyde at a concentration sufficient to provide an aroma which repels pests.

2. The composition according to claim 1, wherein said flavonoid aldehyde is cinnamic aldehyde, α-hexyl cinnamic aldehyde, and/or coniferyl aldehyde.

3. The composition according to claim 1 or claim 2, wherein said concentration is 10-5000 ppm drawn to the total composition.

4. The composition according to any one of claims 1-3, wherein said composition further comprises saponin.

5. A method of repelling pests, e.g. insects, from the vicinity of the body of a mammal; said method comprising topical application of a composition according to any one of the claims 1-4 in an amount which does not cause dermal irritation.

6. The method according to claim 5, wherein said composition is in a form selected from the group consisting of a spray, a stick, a repellant oil and an ointment.

7. The method according to claim 5 or claim 6, wherein said mammal is selected from the group consisting of a human, a bovine, and an ovine.

8. The method according to any one of the claims 5-7, wherein said mammal is a human and said pests are fleas or mosquitos.

9. The method according to any one of the claims 5-7, wherein said mammal is a bovine and said pests are soft ticks.

10. A method of repelling pests from the vicinity of a plant, said method comprising application of a composition according to any one of the claims 1-4, wherein said plant is selected from the group consisting of grape, rose, apple, and corn.

11. The method according to claim 10, wherein said plant is a grape plant and said pests are selected from the group consisting of thrips, nematodes, phylloxera and leaf rollers.

12. The method according to claim 10, wherein said plant is a rose plant and said pests are thrips or melon aphids.

13. The method according to claim 10, wherein said plant is an apple tree and said pests are coddling moths.

14. The method according to claim 10, wherein said plant is a corn plant and said pests are root aphids.

15. A method of preventing an ingress of insects into an area through the application of a composition according to any one of the claims 1-4 to the vicinity of said ingress.

16. The method according to claim 15, wherein said insects are cockroaches.

17. A method of preventing infestations of a tree by pests, said method comprising: contacting the trunk of said tree with a composition according to any one of the claims 1-4.

AMENDED CLAIMS

[received by the International Bureau on 19 September 1996 (19.09.96); original claim 3 cancelled; original claims 1, 4, 5, 8, 10, 15 and 17 amended; new claims 18-21 added; remaining claims unchanged (3 pages)]

1. A composition for repelling pests, said repellent composition comprising at least one flavonoid aldehyde at a concentration sufficient to provide an aroma which repels pests, wherein said concentration is 10-5000 ppm of the total composition.

2. The composition according to claim 1, wherein said flavonoid aldehyde is cinnamic aldehyde, α-hexyl cinnamic aldehyde, and/or coniferyl aldehyde.

4. The composition according to any one of claims 1, 2, and 20, wherein said composition further comprises saponin.

5. A method of repelling pests, e.g. insects, from the vicinity of the body of a mammal; said method comprising topical application of a composition according to any one of the claims 1-2, 4, and 18-20 in an amount which does not cause dermal irritation.

6. The method according to claim 5, wherein said composition is in a form selected from the group consisting of a spray, a stick, a repellent oil and an ointment.

7. The method according to claim 5 or claim 6, wherein said mammal is selected from the group consisting of a human, a bovine, and an ovine.

8. The method according to any one of the claims 5-7, wherein said mammal is a human and said pests are fleas or mosquitoes.

9. The method according to any one of the claims 5-7, wherein said mammal is a bovine and said pests are soft ticks.

10. A method of repelling pests from the vicinity of a plant, said method comprising application of a composition according to any one of the claims 1-2, 4, and

18-20, wherein said plant is selected from the group consisting of grape, rose, apple, and corn, said composition has a phytotoxicity rating of 2 or less.

11. The method according to claim 10, wherein said plant is a grape plant and said pests are selected from the group consisting of thrips, nematodes, phylloxera and leaf rollers.

12. The method according to claim 10, wherein said plant is a rose plant and said pests are thrips or melon aphids.

13. The method according to claim 10, wherein said plant is an apple tree and said pests are coddling moths.

14. The method according to claim 10, wherein said plant is a corn plant and said pests are root aphids.

15. A method of preventing an ingress of insects into an area through the application of a composition according to any one of the claims 1- 2, 4, and 18-20 to the vicinity of said ingress.

16. The method according to claim 15, wherein said insects are cockroaches.

17. A method of preventing infestations of a tree by pests, said method comprising: contacting the trunk of said tree with a composition according to any one of the claims 1- 2, 4, and 18-21.

18. A composition for repelling pests, said repellent composition comprising saponin and at least one flavonoid aldehyde at a concentration sufficient to provide an aroma which repels pests.

19. A composition according to claim 18, wherein said flavonoid aldehyde is selected from the group consisting of cinnamic aldehyde, α-hexyl cinnamic aldehyde, and coniferyl aldehyde.

20. A composition for repelling pests, said repellant composition comprising at least one of α-hexyl cinnamic aldehyde and coniferyl aldehyde, at a concentration sufficient to provide an aroma which repels pests.

21. A method of preventing infestations of a tree by pests, said method comprising: contacting the trunk of said tree with a composition comprising at least one flavonoid aldehyde at a concentration sufficient to provide an aroma which repels pests.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/US 95/17050

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A01N35/02 //(A01N35/02,65:00,25:30)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 8946 Derwent Publications Ltd., London, GB; AN 89-337764 [46] XP002004385 & SE,A,8 900 902 (W.THORSELL) , 13 May 1989	1-3,5-16
Y	see abstract ---	4,17
Y	D.E.H.FREAR: "Chemistry of Insecticides and Fungicides" 1942 , D.VAN NOSTRAND , NEW YORK, US XP002004382 pages 184-191, "WETTING AND SPREADING AGENTS" see page 185, paragraph 4 - page 186, paragraph 1 --- -/--	4

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
5 June 1996	11. 06. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lamers, W

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Week 9418 Derwent Publications Ltd., London, GB; AN 94-144831 [18] XP002004386 & BR,A,9 203 522 (OLIVEIRA BRUM) , 22 March 1994 see abstract	4
Y	DATABASE WPI Week 9109 Derwent Publications Ltd., London, GB; AN 91-061813 [09] XP002004387 & JP,A,03 010 632 (NAGATA) , 18 January 1991 see abstract	17
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Y	see abstract	4,17

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	DATABASE WPI Week 9108 Derwent Publications Ltd., London, GB; AN 91-055734 XP002004391 & JP,A,03 007 554 (HOUSE SHOKUHIN) , 14 January 1991	1-3,5-16
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X	DE,A,36 05 753 (KARAYANNIS GEORGE) 27 August 1987 see claim 1 see column 2, line 25 - line 29	1-3,5-16
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Inter onal Application No
PCT/US 95/17050

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

formation on patent family members

Inter nal Application No
PC:/US 95/17050

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-3605753	27-08-87	NONE	